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Activation of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in Human Lung Microsomes by Cytochromes P450, Lipoxygenase, and Hydroperoxides[1]

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ABSTRACT

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a potent tobacco-specific carcinogen, has been demonstrated to induce lung tumors in animals and is suspected to be a human carcinogen. Cytochromes P450 are the major enzymes responsible for the activation of NNK in microsomes from the lung and liver of rat and mouse, as well as human liver. The present study investigated the enzymes responsible for the metabolic activation of NNK in human lung microsomes. In the presence of a NADPH-generating system, the formation of keto aldehyde and keto alcohol (α-hydroxylation products, measured together), keto acid, hydroxy acid, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol was observed in human lung microsomes. Carbon monoxide (90%) decreased the rate of NNK oxidation by 5–49%, depending on the human lung microsomal samples analyzed. Coumarin decreased the oxidation of NNK by 9–34%, and an antibody against human P450 2A6 decreased the metabolism of NNK by 8–37%, suggesting the involvement of P450 2A6 in NNK oxidation. α-Napthoflavone inhibited NNK oxidation by 6–26%, possibly due to the inhibition of P450 1A1. P450 1A1-expressed microsomes catalyzed the formation of keto aldehyde and keto alcohol, exhibiting K_m values of 1400 μM and 371 μM, respectively. In the absence of NADPH, NNK metabolism resulted in the formation of keto acid, keto aldehyde, and keto alcohol, and the activities in different lung samples were decreased by indomethacin (100 μM; cyclooxygenase inhibitor) or nordihydroguaiaretic acid (100 μM; lipoxygenase inhibitor) by 0–27% or 30–66%, respectively. The addition of arachidonic acid (10–100 μM) increased the rate of the formation of keto aldehyde and keto alcohol approximately 2-fold but inhibited the formation of keto acid. Soybean lipoxygenase increased the rate of formation of keto aldehyde and keto alcohol in a concentration-dependent manner. The increased rate in NNK oxidation by arachidonic acid or lipoxygenase was inhibited completely by nordihydroguaiaretic acid. Catalase, thiourea, and conjugated linoleic acid decreased the rate of NNK oxidation by 47, 20, and 45%, respectively. *tert*-Butyl-hydroperoxide, cumene hydroperoxide, and hydrogen peroxide increased the rate of formation of keto aldehyde and keto alcohol by 210, 40, and 50%, respectively. The results suggest that P450 enzymes are only partially responsible for the activation of NNK in human lung microsomes, and P450 2A6 or a P450 2A6-related enzyme seems to be involved in the activation. Furthermore, lipoxygenase and lipid hydroperoxides may play important roles in the oxidation of NNK in human lung microsomes.

INTRODUCTION

During the processing of tobacco and cigarette smoking, nicotine is nitrosated to form the tobacco-specific nitrosamines NNK,[3] *N'*-nitrosonornicotine, and 4-(methylnitrosamino)-4-(3-pyridyl)butanal (1, 2). Of the tobacco-specific nitrosamines, NNK is the most potent carcinogen and has been suggested to play a role in human tobacco-related cancers (1). It has been demonstrated that NNK induces lung tumors in all laboratory animal species tested (1). For NNK to exert its carcinogenic effect, it must be activated metabolically. Metabolic activation of NNK involves the α-hydroxylation of either the methylene carbon leading to the formation of keto aldehyde and methyldiazohydroxide or the methyl carbon leading to the formation of formaldehyde and 4-(3-pyridyl)-4-oxobutyldiazohydroxide, with the latter compound being a hypothetical precursor for the metabolite keto alcohol (Fig. 1). Generation of the diazohydroxides can result in DNA alkylation and may be responsible for the potent carcinogenicity of NNK (2).

Cytochrome P450 is the most extensively studied enzyme system responsible for the oxidative metabolism of a large number of xenobiotics. P450 has been shown to play a role in the activation of NNK in microsomes prepared from animal tissues and human lung and liver (3–7). We have demonstrated that P450s 1A2, 2A1, and 2B1 or related forms are involved in the oxidative metabolism of NNK in rat (4) and mouse (8) lung microsomes, whereas P450s 1A2 and 2E1 are involved in the formation of keto alcohol in human liver microsomes (7). Using P450s expressed in hepatoma cells or human β-lymphoblastoid cells, P450 1A2 had the highest activity, and P450s 2A6, 2B7, 2D6, 2E1, 2F1, and 3A5 had detectable activity in catalyzing the formation of keto alcohol (7, 9). In a human lung microsomal sample, the activation of NNK was not inhibited by antibodies prepared against P450s 1A2, 2A1, 2C8, 2D1, 2E1, and 3A4 (7). In addition, there is a P450-independent pathway for the activation of NNK in the human lung.

It has been shown that xenobiotics undergo cooxidation to reactive intermediates during arachidonic acid metabolism by way of peroxidative processes (10–15). Prostaglandin synthase and lipoxygenase mediate the oxidation of arachidonic acid for the synthesis of prostaglandins and leukotrienes. These enzymes are present in various organs, including the lung (10, 11, 14). Nonsteroidal antiinflammatory drugs, such as aspirin and indomethacin, inhibit the cyclooxygenase activity of prostaglandin synthase by a competitive and/or irreversible inhibition mechanism (16). Lipoxygenases are inhibited by nordihydroguaiaretic acid, an antioxidant, by reducing the catalytically active ferric lipoxygenase to its inactive ferrous form, resulting in the formation of semiquinone-free radicals (17, 18). Although nordihydroguaiaretic acid is a catecholic antioxidant, it does not inhibit lipoxygenase by trapping enzyme-bound fatty acid radical intermediates (18). Nonsteroidal antiinflammatory drugs have been shown to inhibit NNK-induced lung tumorigenesis and the oxidation of NNK in mouse lung (19, 20). Therefore, it is possible that prostaglandin synthase or lipoxygenase may be involved in the bioactivation of NNK in human lung microsomes. Because NNK may have a potential role in human tobacco-related cancers, it is important to identify the enzymes that are responsible for its activation. The purpose of the present study was to characterize the enzymes that catalyze the oxidation of NNK in human lung microsomes.

MATERIALS AND METHODS

Chemicals. [5-^{3}H]NNK (1.84 Ci/mmol; purity >95%) was purchased from Chemsyn Science Laboratories (Lenexa, KS) and further purified by reverse-

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[3] The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; HPL, human peripheral lung; HPLC, high performance liquid chromatography; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.

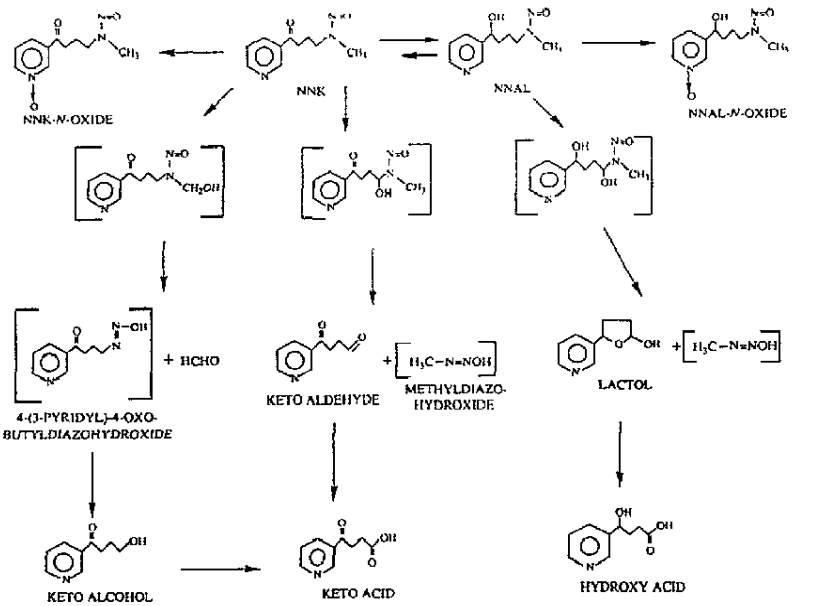


Fig. 1. Metabolic pathways of NNK activation modified from Hecht *et al.* (1).

phase HPLC before use. NNK metabolite standards were supplied kindly by Dr. Stephen Hecht (American Health Foundation, Valhalla, NY). Indomethacin, nordihydroguaiaretic acid, coumarin, α-napthoflavone, troleandomycin, quinidine, arachidonic acid, catalase, superoxide dismutase, conjugated linoleic acid, soybean lipoxygenase (EC 1.13.11.12; type V), cumene hydroperoxide, hydrogen peroxide, and *tert*-butyl-hydroperoxide were purchased from Sigma Chemical Co. (St. Louis, MO). The monoclonal antibody against human P450 2A6, P450 1A1-expressed microsomes, and control microsomes (with vector) were purchased from Gentest Corp. (Woburn, MA). 7-Ethoxycoumarin and 7-hydroxycoumarin were from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade.

Human Lung Microsomes. Human lung tissues were surgical lung resection specimens obtained 0.25–5 h after surgery and stored at −80°C until microsome preparation. The age varied from 16 to 70 years. Of the four samples (denoted HPL with a code number), two were from males, and two were from females. Microsomes were isolated by differential centrifugation, washed, and then resuspended in 0.25 M sucrose (3). Microsomes were stored at −80°C. The protein concentration was determined according to Lowry *et al.* (21), using BSA as the standard. The samples coded HPL 791 and HPL 809 were selected for more detailed studies due to the quantity of microsomes available.

NNK Metabolism Analysis. Unless otherwise stated, the incubation mixtures consisted of 100 mM sodium phosphate (pH 7.4), 10 μM [5-^{3}H]NNK, 1 mM EDTA, 3 mM magnesium chloride, and 0.2 mg microsomal protein in a total volume of 0.2 ml. When a NADPH-generating system was used, it contained 5 mM glucose 6-phosphate, 0.75 units glucose-6-phosphate dehydrogenase, and 1 mM $NADP^+$. The reaction mixture was incubated for 10 min at 37°C and terminated by the addition of 25% zinc sulfate and saturated barium hydroxide. The sample was centrifuged, filtered, and injected onto a reverse-phase HPLC system equipped with a Radiomatic Flo-One/Beta radio flow detector (Radiomatic Instruments and Chemical Co., Tampa, FL; Ref. 3). It was eluted with a linear gradient of 92% A [0.02 M Tris-HCl (pH 6)] and 8% B (95% methanol) to 75% A and 25% B over a 45-min period, followed by a 10-min period of 100% B at a flow rate of 1 ml/min. The identities of the NNK metabolites were determined by coinjecting 5 μl authentic NNK metabolite standards with 100 μl sample onto the HPLC system. In initial experiments, when sodium bisulfite was incorporated into the incubation mixture to trap keto aldehyde directly allowing quantitation of the two α-hydroxylation pathways (22), it coeluted at the same retention time as the keto aldehyde standard in the control blanks at zero time, with boiled microsomes and in the absence of microsomes. Therefore, to eliminate interfering factors, sodium bisulfite was not added to the assay mixtures, and the metabolite peak was quantitated as a mixture of keto aldehyde and keto alcohol. For inhibition studies, the chemical P450 inhibitors were dissolved in methanol and used at 0.5% of the total incubation volume. At this concentration, methanol had no effect on NNK metabolism. All experiments were performed in duplicate.

Arachidonic Acid- or Hydroperoxide-dependent Oxidation of NNK. The assay conditions for arachidonic acid- or hydroperoxide-dependent NNK oxidation were similar to that used for the NADPH-mediated metabolism, except that the NADPH-generating system was replaced by arachidonic acid or the hydroperoxide. To incorporate arachidonic acid or the organic hydroperoxide into the reaction mixture, it was dissolved in methanol, which contributed to 1% of the total incubation volume. At this concentration, methanol did not affect NNK metabolism appreciably.

Coumarin and Ethoxycoumarin Metabolism Analyses. Coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation was determined as described (23). Blanks were incubations without the NADPH-generating system. Fluorescence was measured with $\lambda_{excitation}$ of 358 nm and $\lambda_{emission}$ of 458 nm. The enzyme activities were calculated by comparison with a 7-hydroxycoumarin standard curve subjected to the same incubation and extraction procedures as the samples.

Statistics. Data were analyzed by the Student's *t* test or ANOVA.

RESULTS

NNK Metabolism in Human Lung Microsomes. Under the present experimental conditions, approximately 1 and 5% of the initial amount of NNK were metabolized in the absence and presence of the NADPH-generating system within 10 min, respectively. In the absence of the NADPH-generating system, the metabolism of NNK in the human lung microsomal samples resulted in the formation of keto acid, keto aldehyde, and keto alcohol. The formation of these metabolites was detectable after incubating for 5 min. In longer incubations, additional NNK oxidation still occurred but at a slow rate (Table 1). The rate of NNK oxidation was dependent on the microsomal protein concentration until it reached 0.8 mg (in 0.2 ml; data not shown). In the presence of the NADPH-generating system, the formation of hydroxy acid, keto acid, keto aldehyde, keto alcohol, and NNAL was observed (Table 1). NNAL, the reductive product, was the major metabolite formed and was linear with time. Heat inactivation of the

Table 1 *Time-dependent NNK metabolism in human lung microsomes in the presence and absence of the NADPH-generating system*[a]

Time (min)	Hydroxy acid (pmol/mg protein)	Keto acid (pmol/mg protein)	Keto alcohol and keto aldehyde (pmol/mg protein)	NNAL (pmol/mg protein)
Without NADPH-generating system				
Human lung 791				
0	ND[b]	ND	ND	ND
5	ND	3.26	ND	ND
10	ND	4.26	2.51	ND
30	ND	4.49	3.17	ND
Human lung 809				
0	ND	ND	ND	ND
5	ND	1.56	3.35	ND
10	ND	2.62	4.20	ND
30	ND	3.82	4.26	ND
With NADPH-generating system				
Human lung 791				
0	ND	ND	ND	ND
5	ND	3.74	2.89	232
10	ND	4.06	3.06	397
30	ND	5.03	5.16	1074
Human lung 809				
0	ND	ND	ND	ND
5	1.76	1.72	3.81	93
10	1.87	3.15	5.99	183
30	3.63	4.05	6.19	566

[a] Incubations contained 0.2 mg microsomal protein and 10 μM [5-^{3}H]NNK with or without the NADPH-generating system. The reaction was initiated by the addition of substrate. Incubations were carried out at 37°C. Values are the average of duplicates, and the variation between duplicates was less than 10%.
[b] ND, not detectable.

lung microsomes in boiling water or omission of lung microsomes from the incubation mixtures resulted in no metabolism of NNK (data not shown), indicating that the observed metabolism of NNK is enzymatic. These results suggest that there are NADPH-independent and NADPH-dependent pathways in the oxidation of NNK in human lung microsomes.

Effect of Inhibitors on NNK Metabolism. To determine the contributions of P450 and flavin-containing monooxygenase to the metabolism of NNK in human lung microsomes, carbon monoxide, metyrapone, and preincubation of microsomes at 37°C in the absence of NADPH were used. Carbon monoxide (90%) and metyrapone decreased the rates of formation of hydroxy acid, keto acid, keto aldehyde, and keto alcohol by 5–49%, depending on the particular microsomal sample used (Table 2). The reductive pathway, *i.e.*, the formation of NNAL, was inhibited by 5–38%. In the absence of the NADPH-generating system, the oxidation of NNK was not affected by carbon monoxide and metyrapone (data not shown). Preincubation of lung microsomes at 37°C for 10 min in the absence of NADPH had no effect on the metabolism of NNK (Table 2), suggesting that flavin-containing monooxygenase is not involved in NNK metabolism, or the enzyme is stable during the preincubation in the human lung microsomes. At a concentration of 5 μM, phenethyl isothiocyanate, a compound derived from cruciferous vegetables, which has been shown to inhibit the bioactivation of NNK in mouse lung microsomes by competitive inhibition and chemical inactivation of P450 (3, 8), decreased the rate of NNK oxidation by 44–76% (Table 2). The results suggest that P450 seems to have a partial role in the metabolism of NNK in human lung microsomes.

To determine which P450s are involved in the activation of NNK in human lung microsomes, saturating amounts of α-napthoflavone, coumarin, troleandomycin, and quinidine were used as selective inhibitors of P450s 1A, 2A6, 3A, and 2D6, respectively. α-Napthofla-

Table 2 *Effects of various inhibitors on NNK metabolism in human lung microsomes*[a]

Lung sample code no.	Inhibitor	Hydroxy acid (pmole)	Keto acid (pmole)[b]	Keto aldehyde and keto alcohol (pmol)	NNAL (pmol)
791	Control	ND[c]	0.89 ± 0.04	0.71 ± 0.05	82.3 ± 2.8
	90% carbon monoxide[d]	ND	0.80 ± 0.06 (10)	0.64 ± 0.03 (10)	58.1 ± 2.1[1] (29)
	200 μM metyrapone	ND	0.84 ± 0.02 (6)	0.67 ± 0.05 (6)	75.1 ± 3.9 (9)
	Preincubation at 37°C[e]	ND	0.88 ± 0.01 (1)	0.68 ± 0.02 (5)	81.6 ± 1.5 (1)
	0.5 μM PEITC[f]	ND	0.76 ± 0.05[1] (15)	0.62 ± 0.01 (13)	76.4 ± 2.3 (7)
	5 μM PEITC	ND	0.20 ± 0.01[1] (76)	0.40 ± 0.02[1] (44)	74.8 ± 4.1 (9)
809	Control	0.41 ± 0.03	0.80 ± 0.07	1.13 ± 0.08	44.2 ± 4.1
	90% carbon monoxide	0.21 ± 0.02[1] (49)	0.56 ± 0.01[1] (30)	0.67 ± 0.04[1] (41)	42.1 ± 0.9 (5)
	200 μM metyrapone	0.24 ± 0.01[1] (41)	0.58 ± 0.02[1] (27)	0.80 ± 0.08[1] (29)	45.8 ± 2.1 (0)
	Preincubation at 37°C	0.40 ± 0.02 (2)	0.79 ± 0.01 (1)	1.18 ± 0.05 (0)	48.8 ± 2.5 (0)
	0.5 μM PEITC	0.42 ± 0.03 (0)	0.76 ± 0.05 (5)	0.79 ± 0.05[1] (30)	39.4 ± 3.6 (11)
	5 μM PEITC	0.10 ± 0.01[1] (76)	0.24 ± 0.03[1] (70)	0.52 ± 0.02[1] (54)	37.8 ± 1.1[1] (14)
801	Control	0.60	0.44	0.96	51.2
	90% carbon monoxide	0.40 (33)	0.30 (32)	0.68 (29)	38.2 (25)
802	Control	0.64	0.54	0.90	41.0
	90% carbon monoxide	0.38 (41)	0.40 (26)	0.72 (20)	25.6 (38)

[a] Incubations consisted of 0.2 mg microsomal protein, 10 μM [5-^{3}H]NNK, and a NADPH-generating system. Reactions were carried out at 37°C for 10 min. Numbers in parentheses are percentages of inhibition.
[b] Values are the mean ± SD of four determinations or the average of duplicates. Values with the superscript [1] are significantly ($P < 0.05$) different from the control as determined by the Student's t test.
[c] ND, not detectable.
[d] A mixture of air or carbon monoxide and air (90:10%) was bubbled through the microsome buffer mixture for 3 min.
[e] The microsome buffer mixture was preincubated at 37°C for 10 min in the absence of NADPH.
[f] PEITC, phenethyl isothiocyanate.

Table 3 *Effect of P450 inhibitors on NNK metabolism in human lung 801 microsomes*[a]

Inhibitor concentration (μM)	Hydroxy acid (pmol)	Keto acid (pmol)	Keto aldehyde and keto alcohol (pmol)	NNAL (pmol)
Control	0.32 ± 0.02[b,1]	0.38 ± 0.02[1]	1.24 ± 0.05[1]	43.32 ± 0.76[1]
α-Napthoflavone				
5	0.29 ± 0.02[2] (9)	0.28 ± 0.02[2] (26)	1.24 ± 0.04[1] (0)	45.49 ± 1.94[1] (0)
10	0.30 ± 0.01[2] (6)	0.29 ± 0.03[2] (24)	1.22 ± 0.05[1] (2)	45.22 ± 2.28[1] (0)
Coumarin				
5	0.31 ± 0.01[1] (3)	0.28 ± 0.02[2] (26)	1.13 ± 0.02[2] (9)	45.74 ± 0.66[2] (0)
50	0.27 ± 0.02[2] (16)	0.26 ± 0.01[2] (32)	1.08 ± 0.02[2] (13)	45.35 ± 0.07[1,2] (0)
80	0.28 ± 0.01[2] (13)	0.25 ± 0.03[2] (34)	1.09 ± 0.03[2] (12)	46.76 ± 0.94[2] (0)
TAO[c]				
0	0.37 ± 0.01[1]	0.36 ± 0.02[1]	1.39 ± 0.03[1]	61.65 ± 0.60[1]
20	0.36 ± 0.03[1] (3)	0.34 ± 0.04[1] (6)	1.42 ± 0.04[1] (0)	61.73 ± 0.65[1] (0)
40	0.36 ± 0.04[1] (3)	0.36 ± 0.04[1] (0)	1.38 ± 0.03[1] (1)	62.99 ± 1.39[1] (0)
Quinidine				
2	0.34 ± 0.02[1] (0)	0.35 ± 0.02[1] (8)	1.22 ± 0.02[1] (2)	44.75 ± 1.65[1] (0)
5	0.33 ± 0.02[1] (0)	0.35 ± 0.01[1] (8)	1.23 ± 0.03[1] (1)	45.87 ± 0.21[1] (0)

[a] Incubations contained 10 μM [5-^{3}H]NNK, 0.2 mg microsomal protein, an NADPH-generating system, and inhibitors in a total volume of 0.2 ml. Reactions were carried out for 10 min at 37°C.

[b] Values are the mean ± SD of two replicates. Numbers in parentheses are percentages of inhibition. Values with different superscripts ([1,2]) in the same group are significantly (P = 0.05) different from the control and each other.

[c] Troleandomycin (TAO) was preincubated with microsomes and an NADPH-generating system for 30 min at 37°C before adding the substrate and an NADPH-generating system to start the reaction. Reactions were carried out for 10 min. Values in the TAO group with superscript, [1] are significantly (P = 0.05) different from 0 μM TAO and each other.

vone and coumarin inhibited the oxidation of NNK significantly, by 6–26% and 9–34%, respectively, in human lung (HPL 801) microsomes (Table 3). Similar results were also observed with two other human lung (HPL 791 and HPL 171) microsomal samples (data not shown). Troleandomycin and quinidine had no significant effect on NNK metabolism in any of the human lung microsomes examined. The results suggest that P450s 1A and 2A6 may be involved in pulmonary NNK oxidation.

To investigate further the involvement of P450 2A6 in the metabolism of NNK in human lung microsomes, an antibody against human P450 2A6 was used. The rate of formation of keto acid and keto aldehyde and keto alcohol was decreased by 2–23% and 8–37%, respectively. NNAL formation was inhibited 3–25% by anti-P450 2A6 (Table 4).

Coumarin and Ethoxycoumarin Metabolism. The above results suggest that P450 2A6 or an immunochemically related enzyme is involved in the metabolism of NNK in human lung microsomes. In human liver microsomes, coumarin 7-hydroxylation is a P450 2A6-specific reaction (24, 25). Coumarin (50 μM) was metabolized by the human lung microsomes at a rate of 1.53–2.70 pmol/min/mg protein (Table 5). The human lung microsomal sample (HPL 801), which showed the greatest inhibition by anti-P450 2A6, had the highest activity in metabolizing coumarin. Human lung microsomes catalyzed the formation of 7-hydroxycoumarin from 7-ethoxycoumarin (50 μM) at a rate of 7.56–11.75 pmol/min/mg protein (Table 5), suggesting that other P450 activities in addition to P450 2A6 are present in these human lung microsomes.

Table 4 *Inhibition of NNK metabolism in human lung microsomes by a monoclonal antibody against human P450 2A6*[a]

Sample	Keto acid (pmol)	Keto aldehyde and alcohol (pmol)	NNAL (pmol)
Human lung 801			
Control	0.35 ± 0.02[b]	3.47 ± 0.03	34.3 ± 4.0
Anti-2A6	0.27 ± 0.02[1]	2.18 ± 0.06[1]	27.3 ± 2.5
% inhibition	23	37	20
Human lung 171			
Control	0.35 ± 0.02	1.65 ± 0.03	29.0 ± 1.0
Anti-2A6	0.32 ± 0.03	1.46 ± 0.01[1]	28.3 ± 2.1
% inhibition	9	12	3
Human lung 791			
Control	1.22 ± 0.02	1.81 ± 0.02	56.3 ± 1.5
Anti-2A6	1.19 ± 0.01	1.67 ± 0.04[1]	43.0 ± 1.0[1]
% inhibition	2	8	25

[a] The antibody against P450 2A6 was added to lung microsomes at a concentration of 0.1 μg/μg microsomal protein. The microsome-antibody mixture was kept at room temperature for 15 min before using in incubations. Incubations contained 10 μM NNK, an NADPH-generating system, 0.2 mg microsomal protein, and 20 μg anti-2A6. Reactions were carried out at 37°C for 10 min.

[b] Values are the mean ± SD of two replicates. Values with superscript [1] are significantly (P = 0.05) different from the control as determined by the Student's t test.

Table 5 *Metabolism of coumarin and ethoxycoumarin in human lung microsomes*[a]

Sample	Coumarin 7-hydroxylation (pmol 7-hydroxycoumarin/min/mg protein)	7-Ethoxycoumarin *O*-deethylation (pmol 7-hydroxycoumarin/min/mg protein)
Lung 791	1.53 ± 0.10[b]	7.56 ± 0.29
Lung 171	1.90 ± 0.04	10.00 ± 0.41
Lung 801	2.70 ± 0.03	11.75 ± 0.65

[a] Incubations contained 50 μM coumarin or ethoxycoumarin, an NADPH-generating system, and 0.2 mg microsomal protein. Incubations were carried out at 37°C for 15 min.

[b] Values are the mean ± SD of two replicates.

NNK Metabolism in P450 1A1-expressed Microsomes. Because the results with α-napthoflavone suggested that P450 1A may be involved in the metabolism of NNK, P450 1A1-expressed microsomes were used. The formation of keto aldehyde, keto alcohol, and NNAL was observed with the P450 1A1-expressed microsomes, whereas only NNAL formation was observed with the control microsomes (data not shown). Fig. 2 shows the substrate dependency for the formation of keto aldehyde and keto alcohol in the P450 1A1-expressed microsomes. The apparent K_m and V_{max} values for the formation of keto aldehyde were 1400 ± 148 μM and 151 ± 14 pmol/min/mg protein, respectively. Apparent K_m and V_{max} values of 371 ± 6 μM and 28 ± 4 pmol/min/mg protein were observed for keto alcohol formation.

Effects of Arachidonic Acid and Inhibitors on NNK Metabolism. Fig. 3 demonstrates the arachidonic acid-supported oxidation of NNK. In incubations containing lung microsomes (0.2 mg) and NNK in the absence of NADPH, the formation of keto aldehyde and keto alcohol was increased significantly, approximately 2-fold, by 100 μM arachidonic acid, suggesting that prostaglandin synthase and/or lipoxygenase may be involved. On the other hand, the formation of keto acid was decreased significantly by 15–50% with increasing concentrations of arachidonic acid (Fig. 3).

To determine the contribution of cyclooxygenase and lipoxygenase in catalyzing the formation of keto aldehyde and keto alcohol, indomethacin and nordihydroguaiaretic acid were used, respectively, to inhibit these two enzymes. In incubations without arachidonic acid,

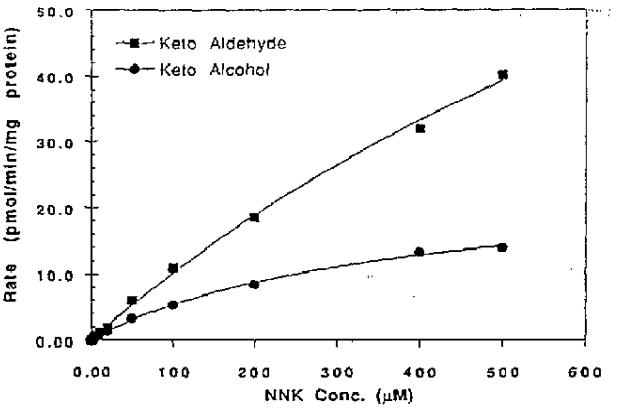
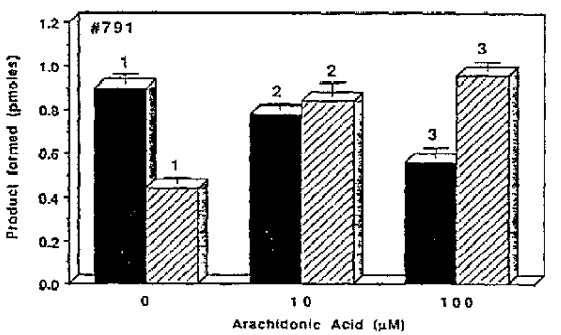


Fig. 2. Substrate dependency for the activation of NNK by P450 1A1-expressed microsomes. Incubations contained an NADPH-generating system, 5 mM sodium bisulfite, 0.2 mg microsomal protein, and 1–500 μM NNK (containing 1 μCi [5-^{3}H]NNK). The P450 content of the microsomes was 34 pmol/mg protein. Reactions were carried out for 30 min at 37°C, and the formation of keto aldehyde (■) and keto alcohol (●) was determined. Values are the mean of two replicates.



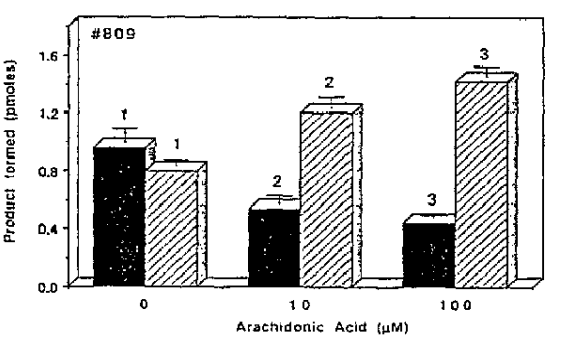


Fig. 3. Arachidonic acid-supported oxidation of NNK in human lung microsomes. Incubations contained 10 μM [5-^{3}H]NNK, 0–100 μM arachidonic acid, and 0.2 mg microsomal protein without NADPH. Reactions were carried out at 37°C for 10 min, and the formation of keto acid (■) and keto aldehyde and keto alcohol (▨) was determined. Values are the mean ± SD (*bars*) of two replicates. Values with different *numbers* for the same metabolite are significantly ($P = 0.05$) different from each other.

indomethacin and nordihydroguaiaretic acid inhibited the rate of NNK oxidation by 0–27% and 30–66%, respectively (Table 6). When arachidonic acid was added to the assay mixture, the rate of keto aldehyde and keto alcohol formation was increased, and the rate was inhibited completely by nordihydroguaiaretic acid, but indomethacin had no clear-cut effect (Table 6). Again, the rate of keto acid formation was decreased by arachidonic acid. The results suggest that lipoxygenase is involved in the formation of keto aldehyde and keto alcohol in human lung microsomes.

Effects of Lipoxygenase on NNK Metabolism. To investigate the involvement of lipoxygenase in the oxidation of NNK in human lung microsomes further, soybean lipoxygenase was added to the incubation mixture. Lipoxygenase displayed a concentration-dependent increase in the rate of formation of keto aldehyde and keto alcohol (Fig. 4). Similar to arachidonic acid, the formation of keto acid was decreased significantly with increasing concentrations of lipoxygenase (Fig. 4). The increased oxidation rate produced by lipoxygenase was inhibited completely by nordihydroguaiaretic acid (Table 7), providing further support for the catalytic activity of lipoxygenase in the formation of keto aldehyde and keto alcohol. On the other hand, lipoxygenase seemed to decrease the rate of keto acid formation.

Effect of Oxygen Radical Scavengers. To determine whether activated oxygen species such as the superoxide anion or hydroxyl radical were involved in the NADPH-independent NNK oxidation in our incubation system, the effects of catalase, superoxide dismutase, and radical scavengers were investigated (Table 8). In the absence of NADPH, catalase decreased the oxidation of NNK significantly, by 34–47%, but superoxide dismutase had no effect. The hydroxyl radical scavenger thiourea decreased the oxidation of NNK by 13–20%. Conjugated linoleic acid, an antioxidant (26), decreased the oxidation of NNK by 33–45%. These results suggest that hydrogen peroxide and the hydroxyl radical are involved partially in the NADPH-independent oxidation of NNK.

Effect of Hydroperoxides. In incubations containing human lung microsomes and NNK without NADPH, the addition of 0.5 mM hydrogen peroxide increased the rate of formation of keto aldehyde, keto alcohol, and keto acid slightly, but a further increase was not produced by higher concentrations of hydrogen peroxide. The addition of cumene hydroperoxide also increased the formation of keto aldehyde and keto alcohol slightly but inhibited the formation of keto acid completely. *tert*-Butyl-hydroperoxide was more effective in increasing the formation of keto aldehyde and keto alcohol than was cumene hydroperoxide, but it was much less effective in decreasing the formation of keto acid (Table 9). The increased formation of keto aldehyde and keto alcohol in the presence of peroxide or hydroperoxides suggests that peroxidative processes are involved.

Table 6 *Effect of arachidonic acid and inhibitors of cyclooxygenase or lipoxygenase on NNK metabolism in human lung microsomes*[a]

Incubation conditions	Keto acid (pmol)	Keto aldehyde and keto alcohol (pmol)
Human lung 791		
Control	0.89 ± 0.02[b]	0.46 ± 0.01
+Indomethacin	0.71 ± 0.04[1] (20)	0.46 ± 0.02 (0)
+Nordihydroguaiaretic acid	0.30 ± 0.04[1] (66)	0.32 ± 0.02[1] (30)
With added arachidonic acid	0.78 ± 0.02	0.87 ± 0.01
+Indomethacin	0.62 ± 0.03[1] (21)	0.76 ± 0.03[1] (13)
+Nordihydroguaiaretic acid	0.52 ± 0.01[1] (33)	0.33 ± 0.03[1] (62)
Human lung 809		
Control	0.86 ± 0.03	0.84 ± 0.03
+Indomethacin	0.63 ± 0.02[1] (27)	0.72 ± 0.02[1] (14)
+Nordihydroguaiaretic acid	0.50 ± 0.03[1] (42)	0.51 ± 0.03[1] (39)
With added arachidonic acid	0.45 ± 0.04	1.32 ± 0.03
+Indomethacin	0.44 ± 0.02 (2)	1.23 ± 0.04[1] (7)
+Nordihydroguaiaretic acid	0.33 ± 0.03[1] (27)	0.39 ± 0.03[1] (70)

[a] Incubations contained 0.2 mg microsomal protein and 10 μM [5-^{3}H]NNK in the presence or absence of 10 μM arachidonic acid. Indomethacin (100 μM) or nordihydroguaiaretic acid (100 μM) was added to reaction mixtures 5 min before incubation. Reaction mixtures were incubated at 37°C for 10 min.

[b] Values are the mean ± SD of two replicates. Numbers in parentheses are the percent inhibition compared with control or arachidonic acid. Values with superscript [1] are significantly ($P = 0.05$) different than the control or arachidonic acid.

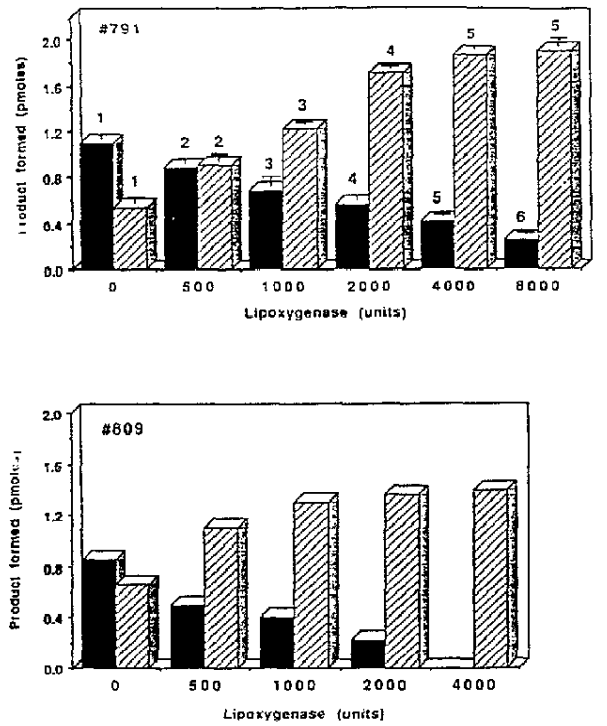


Fig. 4. Effects of lipoxygenase on the oxidation of NNK in human lung microsomes. Incubations contained 10 μM [5-^{3}H]NNK and 0.2 mg microsomal protein in the presence of 0–8000 units soybean lipoxygenase. Reactions were carried out at 37°C for 10 min, and the formation of keto acid (■) and keto aldehyde and keto alcohol (▨) was determined. Values for human lung 791 are the mean ± SD (*bars*) of four determinations. Values for human lung 809 are the average of duplicates. Values with different *numbers* for the same metabolite are significantly ($P = 0.05$) different from each other.

Table 7 *Effect of nordihydroguaiaretic acid on lipoxygenase-catalyzed NNK metabolism in human lung microsomes*[a]

Incubation conditions	Keto acid (pmol)	Keto alcohol and keto aldehyde (pmol)
Human lung 791		
Control	0.94 ± 0.05[b,1]	0.65 ± 0.04[1]
Lipoxygenase	0.73 ± 0.04[2]	1.26 ± 0.05[2]
Lipoxygenase + nordihydroguaiaretic acid	1.00 ± 0.04[1]	0.66 ± 0.04[1]
Human lung 809		
Control	0.79 ± 0.04[1]	0.78 ± 0.04[1]
Lipoxygenase	0.37 ± 0.05[2]	1.43 ± 0.05[2]
Lipoxygenase + nordihydroguaiaretic acid	0.61 ± 0.04[3]	0.81 ± 0.03[1]

[a] Incubations contained 0.2 mg microsomal protein and 10 μM [5-^{3}H]NNK in the presence or absence of 1000 units soybean lipoxygenase; 100 μM nordihydroguaiaretic acid were added to incubation mixtures 5 min before incubating at 37°C for 10 min.
[b] Values are the mean ± SD of two replicates. Values with different superscripts ([1,2,3]) are significantly ($P = 0.05$) different from each other.

DISCUSSION

Metabolism of NNK by human lung microsomes resulted in products derived from α-hydroxylation and carbonyl reduction of NNK. Carbonyl reduction of NNK was the major pathway, and this is consistent with other studies (7, 27). In the present study, P450 accounted for 5–49% of the activity in the oxidation of NNK in human lung microsomes, depending on the samples studied (Table 2). This is different from the situation with rat and mouse lung microsomes (3, 4) and human liver microsomes (7), in which P450 enzymes are fully responsible for the metabolic activation of NNK. Our previous study demonstrated that P450s 1A2, 2A1, 2C8, 2D1, 2E1, and 3A4 were not involved in the oxidation of NNK in the human lung microsomal sample examined (7). P450s 1A1, 2B7, 2F1, 2E1, 3A, and 4B1 are known to be expressed in the human lung (28–33). P450 2A6 has not been reported to occur in the human lung. However, results in the present study with coumarin and anti-P450 2A6 suggest that P450 2A6 or an immunochemically related enzyme is involved in the activation of NNK in the human lung microsomes. 7-Ethoxycoumarin *O*-deethylation has been suggested to be catalyzed by P450s 1A2, 2A6, 2E1, 2F1, and 2B6 (23, 34). The high rates of formation of ethoxycoumarin 7-hydroxylation in the human lung microsomes (Table 5) suggest that human lung microsomes have P450 activities related to one or more of these P450s. Further studies are needed to establish the presence of P450 2A6 in the human lung clearly.

α-Napthoflavone is a selective inhibitor of P450s 1A1 and 1A2. Because P450 1A1 is present in human lung microsomes (30), α-napthoflavone is most likely inhibiting P450 1A1 in the human lung microsomes (Table 3). Although P450 1A1-expressed microsomes catalyzed the formation of keto aldehyde and keto alcohol, a high K_m

Table 8 *Effects of oxygen radical scavengers on NNK oxidation in human lung 791 microsomes*[a]

Reagent	Keto acid (pmol)	Keto aldehyde and keto alcohol (pmol)
Control	1.16 ± 0.08[b,1]	0.60 ± 0.06[1]
40 μg catalase	0.76 ± 0.06[2] (34)	0.60 ± 0.10[1] (0)
80 μg catalase	0.62 ± 0.06[2] (47)	0.36 ± 0.04[2] (40)
5 μg SOD[c]	1.16 ± 0.06[1] (0)	0.62 ± 0.04[1] (0)
10 μg SOD	1.06 ± 0.04[1] (9)	0.56 ± 0.04[1] (7)
10 mM thiourea	0.98 ± 0.04[2] (16)	0.48 ± 0.02[2] (20)
20 mM thiourea	0.96 ± 0.04[2] (17)	0.52 ± 0.08[2] (13)
10 μM CLA	0.66 ± 0.04[2] (43)	0.40 ± 0.04[2] (33)
20 μM CLA	0.64 ± 0.04[2] (45)	0.36 ± 0.02[2] (40)
50 μM CLA	0.64 ± 0.02[2] (45)	0.38 ± 0.06[2] (37)

[a] Incubation conditions in the absence of the NADPH-generating system were as described in "Materials and Methods." Inhibitors were added to reaction mixtures 5 min before incubation. Reaction mixtures were incubated at 37°C for 10 min.
[b] Values are the mean ± SD of triplicates. Numbers in parentheses are percentages of inhibition. Values with different superscripts ([1,2]) in the same group are significantly ($P = 0.05$) different from the control and each other as determined by the Newman-Keuls range test.
[c] SOD, superoxide dismutase; CLA, conjugated linoleic acid

Table 9 *Effect of hydroperoxides on NNK oxidation in human lung 791 microsomes*[a]

Hydroperoxide (mM)	Keto acid (pmol)	Keto aldehyde and keto alcohol (pmol)
Control	0.62	0.33
Hydrogen peroxide		
0.1	0.78	0.40
0.5	0.81	0.50
1.0	0.78	0.48
2.0	0.78	0.49
Cumene hydroperoxide		
0.05	0.14	0.41
0.1	ND[b]	0.46
0.5	ND	0.43
1.0	ND	0.46
2.0	ND	0.47
tert-Butyl hydroperoxide		
0.05	0.47	0.56
0.1	0.37	0.71
0.5	0.28	0.79
1.0	0.14	1.02
2.0	0.14	1.01

[a] Incubations contained 10 μM [5-^{3}H]NNK, 0.2 mg microsomal protein, and 0–2 mM hydroperoxide. Reactions were carried out at 37°C for 5 min. Values are the average of duplicates, and the difference between duplicates was less than 10%.
[b] ND, not detectable.

(1400 μM) was observed for keto aldehyde formation (Fig. 2). Furthermore, the formation of keto alcohol in human lung microsomes was not observed in our previous (7) and present (in the presence of sodium bisulfite) studies. Because humans are exposed to low levels of NNK, the high K_m for keto aldehyde formation by P450 1A1 casts doubt on the significance of this enzyme in the activation of NNK *in vivo*.

The ability of human lung microsomes to metabolize NNK in the absence of NADPH suggests a P450-independent pathway in human lung microsomes. The increased formation of keto aldehyde and keto alcohol by the addition of exogenous arachidonic acid (Fig. 3) or lipoxygenase (Fig. 4), and the inhibition of their formation by nordihydroguaiaretic acid (Tables 6 and 7), suggests the involvement of lipoxygenase in their formation in human lung microsomes. The possible involvement of lipoxygenase in the formation of keto acid also was suggested by results obtained with nordihydroguaiaretic acid (Table 6); however, the addition of arachidonic acid or lipoxygenase decreased its formation. It has been demonstrated that 5-lipoxygenase is inhibited by exogenous arachidonic acid (35). The decrease in keto acid formation may be due to this phenomenon.

The results in the present study, in which the rate of NNK oxidation plateaued at high microsomal protein concentrations (data not shown), and the rate of oxidation was slower after incubation for 5 min (Table 1), are similar to those observed by Egan *et al.* (36) and Nemoto and Takayama (37). The *in vitro* cyclooxygenase-catalyzed reaction for the formation of prostaglandin G_2 is a rapid reaction (36). Furthermore, the lipoxygenase-catalyzed binding of benzo(*a*)pyrene to rat lung microsomal protein was a rapid reaction, which reached a slow phase 4 min after initiation of the reaction, and increased amounts of rat lung microsomal proteins decreased the binding efficiency (37). It has been suggested that the production of oxygen or hydroxyl radicals inhibits the enzymes irreversibly and thereby causes the reaction to plateau (36, 37).

Lipoxygenases exhibit dioxygenase and hydroperoxidase activities (38) and form their hydroperoxide products from arachidonic acid via peroxyl radical intermediates (12). It is during the metabolism of the fatty acid hydroperoxides to alkoxyl radicals that oxidation of xenobiotics can occur. Radicals (peroxyl, hydroxyl, and alkoxyl) also can be generated during microsomal lipid peroxidation, hematin-catalyzed decomposition of fatty acid hydroperoxides, and bisulfite peroxidation or autooxidation (39, 40). Confirmation of the free radical nature of the NADPH-independent oxidation of NNK was provided by the inhibition of NNK oxidation by the antioxidant-conjugated linoleic acid (Table 8). Conjugated linoleic acid can prevent the generation of hydroxyl radicals and thereby may protect membranes against attack by free radicals (26). Furthermore, thiourea inhibited the oxidation of NNK by 13–20% (Table 8), suggesting that hydroxyl radicals are being released into the incubation mixtures as free intermediates. The percent inhibition by thiourea is small, but the involvement of an enzyme-bound radical species as an intermediate (41) is also possible. Recently, it was demonstrated that exposure of MRC-5 fetal human lung cells to NNK resulted in DNA single-strand breaks, which were reduced significantly by superoxide dismutase, mannitol, and catalase, suggesting that oxygen radicals, particularly hydroxyl radicals, were mediating the NNK-induced genetic damage (42). Radicals produced by lipoxygenase via arachidonic acid may be contributing factors in the oxidation of NNK in human lung microsomes.

Usually, microsomal preparations contain catalase activity (43). However, in the present study, the addition of catalase to incubations decreased the oxidation of NNK by 34–47% (Table 8), suggesting that the human lung microsomes contain insufficient amounts of catalase for the disposal of hydrogen peroxide. The decrease in NNK oxidation by added catalase suggests that hydrogen peroxide is involved. Hydrogen peroxide can be generated during enzymatic or nonenzymatic oxidation of a variety of endogenous or exogenous substrates. The hydrogen peroxide that is formed may be used by lipoxygenase in the oxidation of NNK. Kulkarni and Cook (38) demonstrated that hydrogen peroxide could replace fatty acid hydroperoxide in the oxidation of xenobiotics mediated by the hydroperoxidase activity of lipoxygenase.

The involvement of the peroxidase activity of P450 in the presently observed NNK oxidation (Table 9), however, cannot be disregarded completely. P450 functions as a peroxidase by oxidizing compounds when peroxides or hydroperoxides are substituted for NADPH and oxygen in the peroxide shunt (41). However, the lack of inhibition of the NADPH-independent oxidation of NNK by carbon monoxide suggests that the ferrous form of P450 would not be involved in the peroxide-dependent reaction. Other peroxidases, such as myeloperoxidase, also may be involved in the oxidation of NNK. However, further studies are needed to establish the role of peroxidases in the activation of NNK in the human lung.

The results in the present study have important implications for understanding the metabolic activation of NNK in human lung microsomes. It seems that NNK can be activated in human lung microsomes by P450, lipoxygenase and other peroxidases. Although a complete mechanistic description of the lipoxygenase-dependent cooxidation of NNK cannot be made presently, the results suggest that it occurs by a radical-type mechanism. It is possible that peroxyl radical-dependent pathways are more important for activation of carcinogens in tissues with low P450 contents. In the human lung, in which P450 content is low, lipoxygenase and lipid hydroperoxides may have predominant roles in the oxidation of NNK.

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